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Determination of red wine flavonoids by HPLC and effect of aging

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Abstract

A new method for simultaneous determination of 10 flavonols and 2 flavones by high performance liquid chromatography was developed in this paper. The identified compounds contained quercetin, kaempferol, myricetin, rhamnetin, isorhamnetin, quercetrin, rutin, morin, galangin, fisetin, apigenin and luteolin. The chromatographic separation of these flavonoids was performed in a single run by using the mobile phase gradient elution of acetonitrile–methanol–water mixture (1% tetrahydrofuran, THF) at 20 °C, with the flow rate at 1.0 ml/min and the detection wavelength at 360 nm. With direct injection of wine samples, seven red wine samples, differing in their origin of producing places and time, were analyzed for flavonoids content by this method. The results showed the presence of myricetin, luteolin, quercetin, kaempferol, isorhamnetin and galanin. Additionally, the changes of flavonoids in red wines stored in the three types of oak barrels with aging time were investigated, which indicated that the component of flavonoids in red wine is related to wine aging greatly. These provide a substantial basis for the further research on control of flavonoids during winemaking.

Keywords: HPLC; Wine; Flavonols; Flavones; Aging

1. Introduction

In the last decade, polyphenolic compounds have aroused great interest because of their role in assessing the quality of wine (color and taste, etc.) and their importance from a medical point of view (antioxidant, antitumoral and against coronary heart disease (CHD), etc.) (Gronbaek et al., 1995; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993a; Knekt et al., 1997; Renaud & de Lorgeril, 1992). These compounds can be classified into two kinds: flavonoids and nonflavonoids (Frankel, 1996; Hertog, Hollman, & Van de putte, 1993b). Flavonoids are a large family of over 4000 ubiquitous secondary plant metabolites, which can be further divided into five subclasses including flavonols, flavones, anthocyanins, catechins and flavonones (Merken & Beecher, 2000). Flavonols such as quercetin, myricetin, isorhamnetin, kaempferol and the corresponding flavones, apigenin and luteolin have been well established as potent antioxidants that prevent oxidant of low-density lipoprotein and inhibit lipid peroxidation (Formica & Regelson, 1995; Hertog & Hollman, 1996; Shahidi & Wanasundara, 1992). Evidently, different flavonoids of wines are of biological interest to a different extent. Considering the importance of biologically active flavonoids of wines, it is thus necessary to develop an accurate and rapid method for the analysis of flavonoids.

Determination of flavonols and flavones in food and beverages has been reported by means of various methods including thin layer chromatography, high-performance liquid chromatography (HPLC), gas chromatography and capillary electrophoresis (CE). HPLC analysis revealed that quercetin, myricetin and kaempferol are the major flavonol aglycones in wines (McDonald et al., 1998; Tsanova-Savova & Riharova, 2002) and apigenin and luteolin (Hertog et al., 1993b; Wang & Huang, 2004)and isorhamnetin (Garcia-Viguera & Bridle, 1995) are also present in wines. Recently, a new HPLC method was established for simultaneously determining six flavonoids containing quercetin, myricetin, kaempferol, apigenin, luteolin and galagin (Wang & Huang, 2004). Here, we developed a HPLC

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method by which 10 flavonols and 2 flavones can be well identified in a single experiment. These compounds contain quercetin, kaempferol, myricetin, rhamnetin, isorhamnetin, quercetrin, rutin, morin, galangin, fisetin, apigenin and luteolin. On this basis, we investigated the changes of flavonols and flavones contents in red wine stored in three different types of oak barrels with the aging time since aging in oak barrels is a very important step in red wine manufacture and great physical and chemical changes had taken place during this period (Fulcrand, Doco, Es-Safi, Cheynier, & Moutounet, 1996; Somers, 1971; Zafrilla et al., 2003). These might provide a substantial basis for the further research on control of flavonoids during winemaking.

2. Materials and methods

2.1. Wine samples

All the commercial wine samples were supplied by Chinese famous wine producers, as listed in Table 1.

All the aging wine samples and oak barrels were supplied by Huaxia Winery Co. Ltd., (Changli, Hebei, China). The wine brewing process strictly obeyed the manufacture technics of red wine made from Carbernet Sauvignon in China Huaxia Winery Co., Ltd., in 2004 and was subsequently aged during a period of 135 days in new barrels of European oak barrels (*Quercus petraea*), American oak barrels (*Quercus alba*) and occident oak barrels. All the barrels were made at Seguin Moreau Napa Cooperage (France). For the manufacture of the barrels, the wood was naturally seasoned for 36 months and all of the barrels were submitted to a medium toasting. Wine aging samples to be analyzed were taken from the barrels after 0, 30, 60, 90, 105, 120 and 135 days of aging. In all cases, the wine was put into three barrels of each type of oak and consequently, all the experiments were made in duplicate. One representative sample was taken from each of the barrels, and the flavonols and flavones from each sample were analyzed in duplicate. Results presented in the figures are showed with their standard deviations and are the arithmetic mean of three analyses.

2.2. Standards

Flavonoid standards, including apigenin (10798), isothamnetin (17794), rhamnetin (17799), fisetin (46340), galangin (48291), kaempferol (60010), luteolin (62696), myricetin (70050) were purchased from Fluka (Buchs, Switzerland), morin (M4008), quercetin dihydrate (Q0125), quercitrin (Q3001), rutin hydrate (R5143) were purchased from Sigma Chemical Co. (St. Louis, USA). The purities of the 12 analytes were up to 95%. All the standards were dissolved in methanol to a concentration of 1 mg/ml and were stored in darkness at −20 °C. All the standard solutions proved to be stable for over 3 months. The solvent of tetrahydrofuran (THF), acetonitrile and methanol, labeled as HPLC grade were purchased from Fisher Scientic (USA).

2.3. Preparation of samples

Without any extraction and hydrolysis, all the wine samples were filtered through a 0.45 μm filter for organic solvents (Acrodisc LC13 PVDF filter, Gelman/Pall Life Sciences, MI) prior to the injection of 40 μl to HPLC analysis.

2.4. High-performance liquid chromatography

Chromatographic separations were performed on a Merck LiChrospher 100RP-18e (Merck, Germany) column (250 × 4.0 mm ID, 5 μm), protected by a Merck RP-18 (10 mm × 4.0 mm) guard column. Both columns were placed in a column oven set at 20 °C. The HPLC system consisted of Shimadu (Japan) LC-6A series pumping system, SIL-6A automatic injector furnished with a 50-μl loop, SPD-6AV UV–visible detector set at 360 nm and C-R6A chromatography data station software. Two solvents were used with a constant flow rate of 1.0 ml/min. Solvent A consisted of 19% acetonitrile, 5% methanol and 1% THF in water (pH 3.0), solvent B included 55% acetonitrile and 15% methanol in water(pH 3.0). All the solvents used were of HPLC grade. For the elution program, the following proportions of solvent B were used: 0–15 min, 2% B; 15–28 min, 2–28% B; 28–40 min, 28–36% B; 40–44 min, 36% B; 44–45 min, 36–80% B; 45–52 min, 80% B.

3. Results and discussion

3.1. High-performance liquid chromatography

3.1.1. Detection wavelength

The maximal absorbance wavelengths (λ_{max}) of the 10 flavonols and 2 flavones were analysed by scanning between 200 and 400 nm on the Shimadu UV-2405 in order

Table 1
List of wine samples

Wine	Wine producer	Vintage year
Hua-xia Zone A red wine	Huaxia Winery Co. Ltd. (Changli, Hebei, China)	Zone A* (2000)
Hua-xia Great-Wall red wine	Huaxia Winery Co. Ltd. (Changli, Hebei, China)	1999
Hua-xia Great-Wall red wine	Huaxia Winery Co. Ltd. (Changli, Hebei, China)	1995
Hua-xia Great-Wall red wine	Huaxia Winery Co. Ltd. (Changli, Hebei, China)	1994
Hua-xia Great-Wall red wine	Huaxia Winery Co. Ltd. (Changli, Hebei, China)	1992
Dragon-seal red wine	Dragon-seal Wines Co. Ltd. (Beijing, China)	2001
Dragon-seal red wine	Dragon-seal Wines Co. Ltd. (Beijing, China)	2002

* As a special grape planting zone of Huaxia Winery Co. Ltd. (Changli, Hebei, China).

to obtain the optimal detection wavelengths applied for the chromatography separation. Spectra chromatography showed that the absorbance peaks of the 12 flavonoids were different, but they all had the best or better absorbance peak at 360 nm, therefore, the UV–vis detector was set at 360 nm in this method.

3.1.2. Optimization of mobile phase

In this method, the above mobile phase and the Linear gradient elution program system were tested by separating the 12 flavonoid standards, the result showed that the method can successfully separate 12 flavonoids and the chromatography of 12 flavonoid standards in this system was shown in Fig. 1.

Methanol and acetonitrile were the most widespread chromatographic mobile phase in the investigations of flavonoids at present. In this experiment, we found that by increasing the proportion of methanol in the mobile phase properly, not only the standard peaks appeared ahead of time but also the shape of the peaks appeared much more symmetrically, which was accordant with the result before (Yu & Xu, 1997). Besides, due to the similar structure and biological properties of flavonols and flavones in wine, we had met a lot of difficulties in the separation and quantification of them, by adding 1% THF in solvent A, the problems appeared before were solved successfully, according to the result of Wang and Huang (2004). Easily ionizing ability of the phenolic hydroxyl group makes the tailing phenomenon at the end of the standard peaks, by adding phosphoric acid, we controlled the aqueous at pH of 3, which helped separate all the standard peaks successfully.

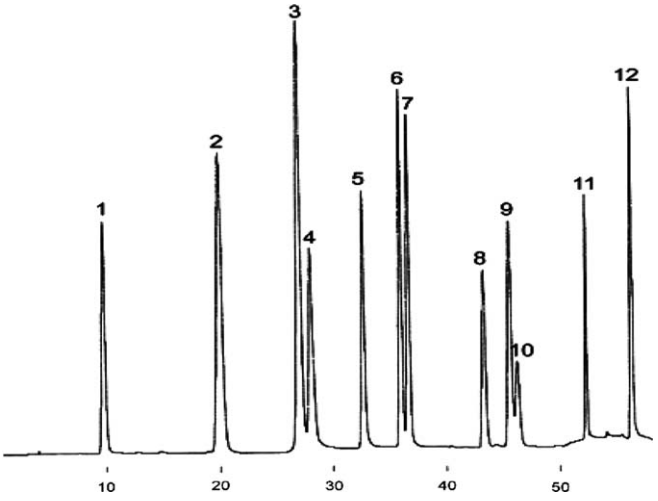

Fig. 1. Chromatogram of 12 Flavonoids standards: (1) Rutin; (2) quercetrin; (3) fisetin; (4) myricetin; (5) morin; (6) luteolin; (7) quercetin; (8) apigenin; (9) kaempferol; (10) isorhamnetin; (11) rhamnetin; and (12) galanin.

3.1.3. Establishment of the calibration curves

The calibration curve of each flavonoids was established by injecting 5 different concentrations of the standard mixtures consisting of 10 flavonols and 2 flavones (Table 2). Other results, such as retention time, limit of detection (LOD), linear range and correlation coefficient (R) were also listed in Table 2. Results showed that the LOD of each flavonoid is very low (1.00×10^{-2}–3.60×10^{-2} mg/L), which indicated that this method had a high degree of sensitivity.

3.1.4. Reproducibility of HPLC analysis

The reproducibility of the HPLC analysis was carried out in two ways, i.e., retention times and peak areas. In this method, the coefficient of variation (CV) for the reproducibility of the HPLC analysis was obtained through five injections of the same standard mixture and the results were listed in Table 3. The CV for the retention time of all peaks of the standards was <2% and the CV for the peak area was <5%, which indicated that this method was of good reproducibility and were acceptable.

3.2. Quantitative of flavonoids in grape wine

Seven kinds of red wine made in China were analyzed by the developed HPLC method, they were from two famous wine producers in China and of different ages, the flavonoids contents were listed in Table 4. The results showed that the major constituents of flavonoids in red wine is quercetin (0.17–4.87 mg/L) and myricetin (1.57–4.45 mg/

Table 2
Quantitative results of the studied flavonols and flavones by HPLC

Compounds	Retention time (min)	Linear range (mg/L)	Calibration curve	Correlation coefficient (R)	Detection limit (mg/L)
Rutin	9.833	6.58–105	$Y = 26252x - 53064$	0.9997	2.34×10^{-2}
Quercetrin	19.85	2.65–42.50	$Y = 74810x - 131283$	0.9992	3.60×10^{-2}
Fisetin	26.767	1.55–24.80	$Y = 69884x - 72806$	0.9991	1.46×10^{-2}
Myricetin	27.783	1.26–20.20	$Y = 61614x - 10521$	0.9991	2.53×10^{-2}
Morin	32.467	7.12–114	$Y = 897.9x - 2666.1$	0.9994	2.30×10^{-2}
Luteolin	35.783	0.15–24.80	$Y = 47153x - 20994$	0.9988	1.74×10^{-2}
Quercetin	36.458	0.26–42.80	$Y = 26232x - 25927$	0.9982	1.62×10^{-2}
Apigenin	43.163	2.45–39.10	$Y = 21231x - 20931$	0.9992	2.78×10^{-2}
Kaempferol	45.467	0.23–37.90	$Y = 31832x - 28017$	0.9984	2.11×10^{-2}
Isorhamnetin	46.135	0.12–19.40	$Y = 37986x - 23505$	0.9982	2.88×10^{-2}
Rhannetin	52.158	0.62–9.90	$Y = 50892x - 17973$	0.9996	1.00×10^{-2}
Galangin	56.178	0.14–23.80	$Y = 44374x - 20756$	0.9985	1.30×10^{-2}

Table 3
Reproducibility of the studied flavonols and flavones of HPLC ($n = 5$)

Compounds	Retention time (min)	Retention time CV (%)	Peak area CV (%)
Rutin	9.833	0.840	1.8
Quercetrin	19.85	0.729	1.4
Fisetin	26.767	0.733	2.1
Myricetin	27.783	0.724	2.5
Morin	32.467	0.752	3.4
Luteolin	35.783	0.855	2.6
Quercetin	36.458	0.801	1.4
Apigenin	43.163	1.06	2.0
Kaempferol	45.467	1.07	1.9
Isorhamnetin	46.135	0.835	5.0
Rhamnetin	52.158	0.371	1.3
Galangin	56.178	0.801	3.6

L), the minors are luteolin (0.18–0.96 mg/L), kaempferol (0.06–0.20 mg/L), isorhamnetin (0.03–0.54 mg/L) and galangin (0.01–0.04 mg/L), while the other six flavonoids (rutin, quercetrin, fisetin, morin, apigenin, rhamnetin) were not detected in these wine. According to the wine sample results, it is obviously that the contents of flavonols and flavones of wines coming from different wine producers and of different ages were totally different, this may be related to the thickness of the grape skin, the climate that the grape grows in, the ripe degree of the grape, the application of different modern methods of vinification and the wine ages (McDonald et al., 1998) (see Fig. 2).

3.3. Changes of flavonol and flavone contents during wine aging in oak barrels

Using the above HPLC method, aging wine samples collected from three different types of oak barrels were analyzed respectively after 0, 30, 60, 90, 105, 120 and 135 days of aging. Fig. 3 shows that the contents of six flavonoids of red wine changed with aging time in oak barrels and there was more or less difference in their change patterns among three types of oak barrels (European, American and accident oak barrels). During aging in oak barrels from 0 to 135 days, significant changes in the contents of these flavonoids were observed in all the used oak barrels. Changes of the contents of myricetin and isorhamnetin presented fluctuant (Fig. 3A and E), and the contents of luteolin gradually decreased until the end of oak barrel aging (Fig. 3B). Quercetin content started to decrease rapidly within 60 days after the onset of aging and then slight increased (Fig. 3C). It is noteworthy that galanin was not detected until 135 days of wine aging in oak barrels, indicating that galanin in red wine derived from oak wood (Fig. 3F). For kaempferol, change pattern in European oak barrel was significantly different from that in American oak barrel and in occident oak barrel, which indicated that both aging time and oak species affected the component of the finished red wine. Among these three types of oak barrels, changes of other five flavonoids, except for kaempferol, presented similar patterns with aging time.

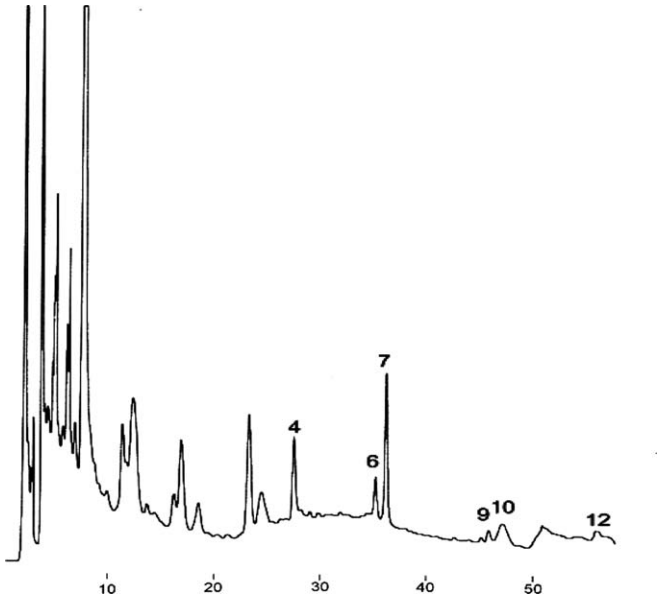

Fig. 2. Flavonols and flavones compounds in wine sample: (4) Myricetin; (6) luteolin; (7) quercetin; (9) kaempferol; (10) isorhamnetin; and (12) galanin.

In all, the reduction in the concentrations of myricetin, luteolin, quercetin and kaempferol after aging in the three different types of oak barrels could be due to the combination of sugar and flavonol aglycones to form the flavonol glycosides (Hertog & Hollman, 1996). While with more and more flavonol glycosides had been formed during wine aging, the flavonol glycosides themselves were hydrolyzed into flavonol aglycones (Somers, 1971) in the oak barrels,

Table 4
Content of flavonols and flavones in 7 different wine samples ($n = 3$)

Compounds	Hua-xia 1999	Hua-xia 1995	Hua-xia 1994	Hua-xia 1992	Hua-xia Zone A	Dragon-seal 2002	Dragon-seal 2001
Myricetin (mg/L)	3.96 ± 0.39	3.13 ± 0.18	2.84 ± 0.31	4.45 ± 0.39	2.77 ± 0.24	2.77 ± 0.12	1.57 ± 0.14
Luteolin (mg/L)	0.28 ± 0.02	0.25 ± 0.02	0.24 ± 0.01	0.20 ± 0.02	0.18 ± 0.01	0.96 ± 0.04	0.43 ± 0.04
Quercetin (mg/L)	0.72 ± 0.08	0.22 ± 0.07	0.17 ± 0.01	4.87 ± 0.41	1.37 ± 0.13	2.65 ± 0.20	1.75 ± 0.20
Kaempferol (mg/L)	0.20 ± 0.01	0.16 ± 0.01	–*	0.15 ± 0.02	0.20 ± 0.02	0.08 ± 0.00	0.06 ± 0.02
Isorhamnetin (mg/L)	0.13 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.54 ± 0.06	0.19 ± 0.01	0.46 ± 0.02	0.30 ± 0.06
Galangin (mg/L) $\times 10^{-2}$	1.30 ± 0.58	1.30 ± 0.14	1.60 ± 0.14	1.40 ± 0.29	4.10 ± 0.58	–*	2.00 ± 0.10
Total content (mg/L)	5.30 ± 0.46	3.80 ± 0.27	3.29 ± 0.32	10.22 ± 0.41	4.75 ± 0.35	6.93 ± 0.32	4.13 ± 0.14

Data in the table are the means of three replicates.
* Not detected.

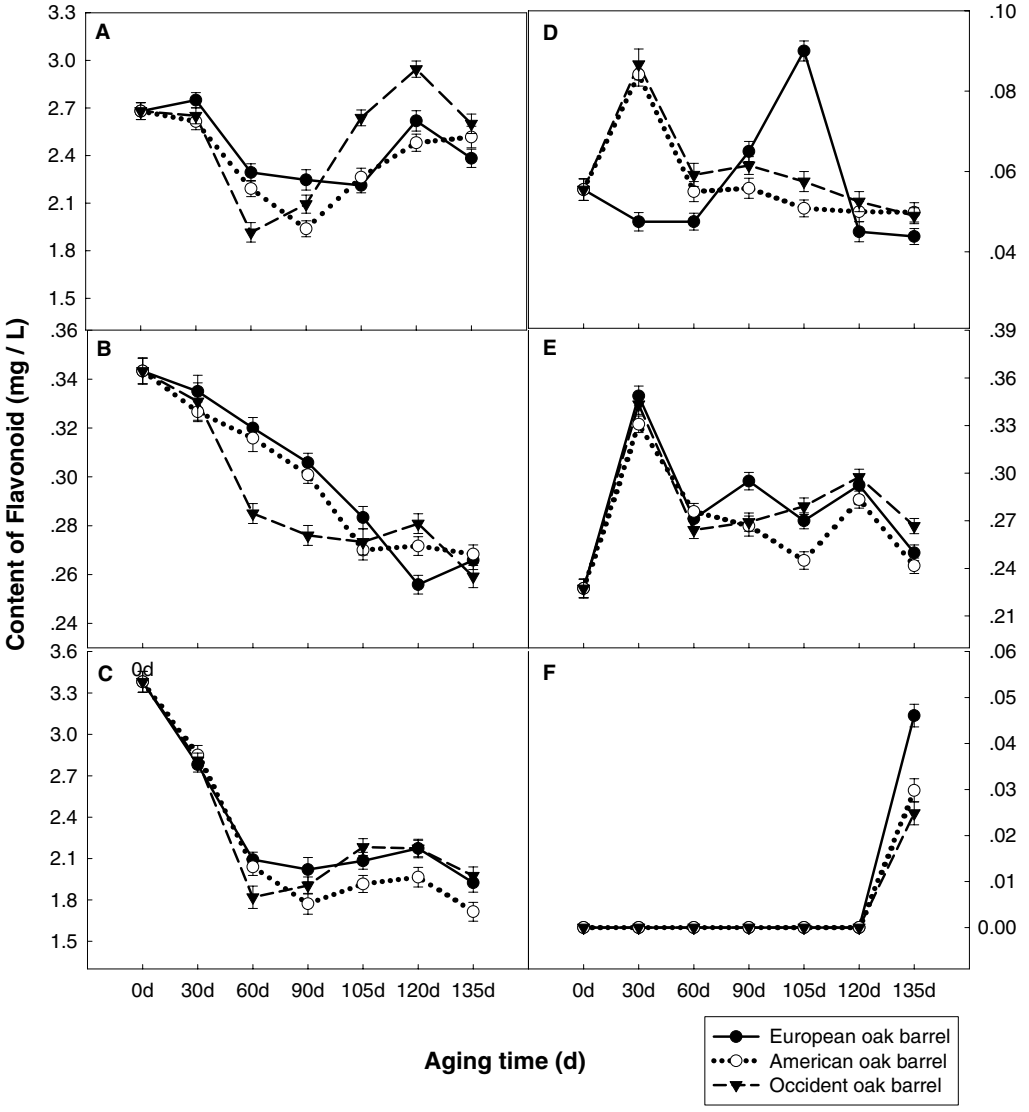

Fig. 3. Comparation of the evolution of each type of flavonols or flavones with the aging time among three types of oak barrels: (A) Myricetin; (B) luteolin; (C) quercetin; (D) kaempferol; (E) isorhamnetin; and (F) galanin.

which could explain why the flavonol concentrations waved with the wine aging time. While according to the structure of isorhamnetin (4′-methoxy-quercetin) in nature, the increase in the concentration of isorhamnetin after wine aging in oak barrels was probably due to the transformation of quercetin to isorhamnetin in oak barrels, which have great impact on the redox happened in wine. Besides, the appearance of galanin at the end of the aging time also sent us a notable signal that the trace concentration of galanin in wine maybe came from the oak barrels directly or was one of the outcomes between wine and the oak barrels, while not came from the grape fruit directly.

4. Conclusion

The present study developed a new method for simultaneous determination of 10 flavonols and 2 flavones by RP-HPLC without sample pretreatment. The results showed that this new method was simple, practicable, and feasible with high precision, sensitivity and repeatability and could also provide a good resolution of the major flavonols and flavones in wines.

The results of the wine samples showed that quercetin, myricetin were the main flavonols aglycones in wines, while luteolin, keampferol, isorhamnetin and galagin were also present in wine in low content. Moreover, as a result of difference in climates and grape ripeness, the application of vinification, wine manufactures and wine ages, concentrations of flavonols and flavones of those wines were also different. Although no other new flavonoids were detected in wines, while this new method had offered a new way to detect the flavonoids content in other foods.

On the basis of the developed method, it was investigated for the first time, to our knowledge, about the evolution of flavonols and flavones contents in wine with wine aging time, and the impacts of three different types of

oak barrels on wine aging. And galanin was also observed for the first time at the end of wine aging in three different oak woods, which indicated that the origin of galanin in wine was related to wine aging in oak barrels greatly.

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